AFFINITY OF LIGNIN PREPARATIONS TOWARDS GENOTOXIC COMPOUNDS

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The carcinogenicity and mutagenicity of chemicals may be modulated by other chemicals, including those prepared by organic synthesis. Considering the several drawbacks of synthetic compounds vis-a-vis the human organism, the lignin biomass component was examined for this purpose. The binding affinity of lignin samples prepared by chemical and biological modification of lignin products derived from chemical wood treatment towards for N-nitrosodiethylamine (NDA) was examined. The protective role of the lignin samples against carcinogenesis was tested on a well-known model carcinogen, N-methyl-N´-nitro-N-nitrosoguanidine (MNNG). The observed ability of a series of lignin preparations to reduce alkylation damage of deoxyribonucleic acid (DNA) on hamster cells in vitro could be explained by their affinity to bind N-nitrosoamines. The results indicate that lignin has potential to protect living organisms against damaging effects of different genotoxicants.

Keywords: Lignin adsorbents; Modification; G. Klebahnii; S. Roseus; NDA; MNNG;Genotoxic compounds

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INTRODUCTION

Lignin belongs to the most abundant group of organic polymers on earth. The renewable lignocellulosic resources contain 17-33 % lignin by weight. Lignin is obtained as a co-product of pulp production. The elementary unit of the lignin macromolecule is a phenyl propane C_9-unit. The structure of aromatic and side-chain parts varies due to different genetic origin of wood as well as modification of lignin during thermal and chemical treatment. In view of its natural origin, our research has been focused on the conversion of lignin waste products from the pulp and paper industry into useful products, such as pulping additives (Košíková et al.1990), azo-dye dispersants (Košíková and Demianová 1995), etc. Our previous study of the antimicrobial effect of lignin preparations showed that they inhibit the growth of well-known pathogenic yeasts (Košíková and Sláviková 1998). The antimicrobial properties of lignin were confirmed also by the study of Lora and Glasser (2002).

In our previous paper (Košíková et al. 1984), it was revealed that non-modified kraft lignin exhibits very low affinity for bile acid adsorption. It could be significantly enhanced by chemical modification. The aim of the present work was examination of modified lignin preparations for utilization as nitrosoamine adsorbents. Genotoxic chemicals such as nitrosoamines occur naturally in the diet and can be produced in food processing, baking, cooking, and storage, or by reaction in the gastrointestinal tract. The potential protective role of lignin biomass components against carcinogenesis was

investigated by the characterization of a binding affinity of lignin preparations towards the well-known carcinogen N-nitrosodiethylamine (NDA). Moreover, the antimutagenic effect of lignin samples on MNNG-induced DNA damage *in vitro*, as well as on halogenated hydrocarbon pesticide DBCP-induced mutagenic lesion *in vivo*, was examined. Drawing on our knowledges about the ability of some yeast species to biotransform the lignin macromolecule (Košíková and Sláviková 2004), besides of chemical modification, also biological treatments with the yeast strains *Sporobolomyces roseus* and *Geotrichum klebahnii* were used for modification of polymeric lignin products isolated from wastes of pulp and paper industry in order to prepare natural adsorbents of genotoxic chemicals.

EXPERIMENTAL

Kraft lignin was precipitated from concentrated spruce kraft liquors (total solids 59.9 %, Klason lignin 32.7 %, ash 27.2 %). The precipitates were filtered, washed, and dried. Chemical treatment of this lignin with diluted sulphuric acid (60 % w/v) yielded the preparation (lignin 1) having average molecular mass 8800 with 15.2 % OCH_3 and 6.5 % phenolic OH. The modification of beech wood prehydrolysis lignin with yeast species *Geotrichum klebahnii* and *Sporobolomyces roseus* yielded lignin 2 (average molecular mass 1850, 13.7 % OCH_3, 4.5 % phenolic OH) and lignin 3 (average molecular mass 1930, 12.3 % OCH_3, 4.1 % phenolic OH), respectively.

The yeast strains were obtained from the Culture Collection of Yeasts (Institute of Chemistry, Slovak Academy of Sciences, Bratislava). These strains were cultivated in a medium containing 6.7g yeast nitrogen base (Difco) and lignin sample (3g) per liter of solution in distilled water.

Hydrolysis lignins were prepared by refluxing of pre-extracted spruce and beech sawdust as well as disintegrated straw with sulphuric acid (60 % w/v) for 7 h. The undissolved residue was filtered off, washed with water, and dried (yields 20-30 %). Modification of hydrolysis lignins was performed by treatment with 0.01 M NaOH (1 h, 25^{o}C).

Gel permeation chromatography was performed on a column (53 x 0.8 cm) of Sephadex LH 60 using a mixture of dioxane and water (7:3) containing 0.005 M aqueous NaOH and 0.001 M LiCl as the eluant (Košíková et al. 1990). Phenolic hydroxyl groups were determined by FTIR spectroscopy (Faix et al. 1992).

N-nitrosodiethylamine (NDA) binding on lignin was made by dispersing 0.5 g of lignin in phosphate buffers (pH 5.4 and 8.0), with the nitrosoamine added (3.10^{-3} $M.L^{-1}$), the total volume being 25 ml. This method is based on the determination of NDA concentration remaining in the solution after an addition of lignin preparations. The dispersion was shaken at 25^{o}C for 17 h. The NDA concentration in the supernatans was determined by using a universal polarographic analyzer OH-105. The desorption test was performed by suspending of the lignin samples with adsorbed NDA in 1.10^{-2} M NaCl at pH 5.4, and shaking for 24 h.

For NDA binding experiments on polysaccharides holocellulose was prepared by delignification of spruce wood according to Klauditz (1957) at 40°C for 40 h, as well as microcrystalline cellulose and xylan from birch wood purchased from Sigma-Aldrich Chemie, Germany.

A sample of quasidiploid Chinese hamster V79 cells was obtained from Dr. A. Abbondandolo (University of Genoa). The hamster cells were preincubated with a lignin sample dissolved in dimethyl sulfoxide (50 μg ml^{-1}) at 37°C for 120 min prior to treatment with MNNG (2 μg/ml). The cells were treated with MNNG without preincubation or after preincubation with lignin for 2 h. The level of DNA damage (DNA strand breaks) was measured using single-cell gel electrophoresis, i.e., a comet assay. The genotoxicity of lignin samples was tested in V79 cells. The level of DNA strand breaks for all lignins prepared was comparable with the level of breaks in control cells, indicating that incubation with lignins had no genotoxic effect on cells. The re-spreading mutation assay proposed by Chasin (1973) was used for detection of 6-TG resistant mutations. The male Sprague –Dawley rats obtained from ANLAB, Czech Republic were housed two per cage under standard environmental conditions (22 ± 2°C, 55 ± 5% relative humidity, and lights on from 06:00 to 18:00 h) in solid plastic cages on hardwood bedding. The rats were fed daily by a standard diet or a lignin-supplemented diet (10 g/100 g body wt/day) for 21 days. Rat primary hepatocytes were isolated from rats using an *in situ* two-step collagenase perfusion technique as described by Michalopoulos et al. (1982) with some modifications (Eckl et al. 1987). After isolation the cells were kept in complete minimal essential medium (MEM) containing 1.8 mmol calcium. 1,2-dibromo-3-chloropropane (DBCP) was prepared by bromination of allyl chloride (Omichinski et al. 1987). DBCP was diluted in DMSO to concentrations 10 and 30 μmol/l shortly before use and kept at 4°C.

All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

The results summarized in Table 1 showed that lignin adsorbents prepared by chemical and biological modification exerted a high binding affinity for N-nitrosodiethyl-amine at pH 5.4 and 8.0.

Table 1. Binding of N-nitrosodiethylamine (NDA) to Lignins Dispersed in 1.10^{-2} M NaCl (20 $mg.mL^{-1}$)

Sample	Adsorbed NDA %	
	pH 5.4	pH 8.0
Lignin 1	58.5	53.8
Lignin 2	52.1	49.2
Lignin 3	53.6	51.8

The adsorption of NDA to lignins was almost complete in about 5 h. When samples of lignin with adsorbed NDA were separated from the supernatant, suspended in 1.10^{-2} M NaCl at pH 5.4, and shaken for 24 h, about 50 % of NDA was desorbed. This

indicates that the prepared lignin samples could be effective in the gastrointestinal tract. In contrast with lignin, all tested carbohydrate preparations (microcrystalline cellulose, holocellulose, and xylan) were poor adsorbents for N-nitrosodiethylamine. The obtained results were in agreement with observation of Rubio and Falkehag (1979), indicating that lignin has high binding affinity towards NDA. The other dietary fiber components tested were less effective for nitrosoamine than lignin.

In our further experiments, the binding capacities of hydrolysis lignins from spruce wood, beech wood, and straw were determined. As is shown in Fig. 1, the obtained data varied with the genetic origin of lignins (Table 1). This observation could be explained by the different ratios of p-hydroxy phenyl propane, guaiacyl, and syringyl units in the starting material. It is known (Sarkanen and Ludwig 1971) that spruce wood, as for the majority of gymnosperms, contains mostly guaiacyl lignin. Beech wood lignin is a copolymer of guaiacyl and syringyl units (57:43). Straw lignin is a copolymer of p-hydroxy phenyl, guaiacyl, and syringyl units (31:28:41).

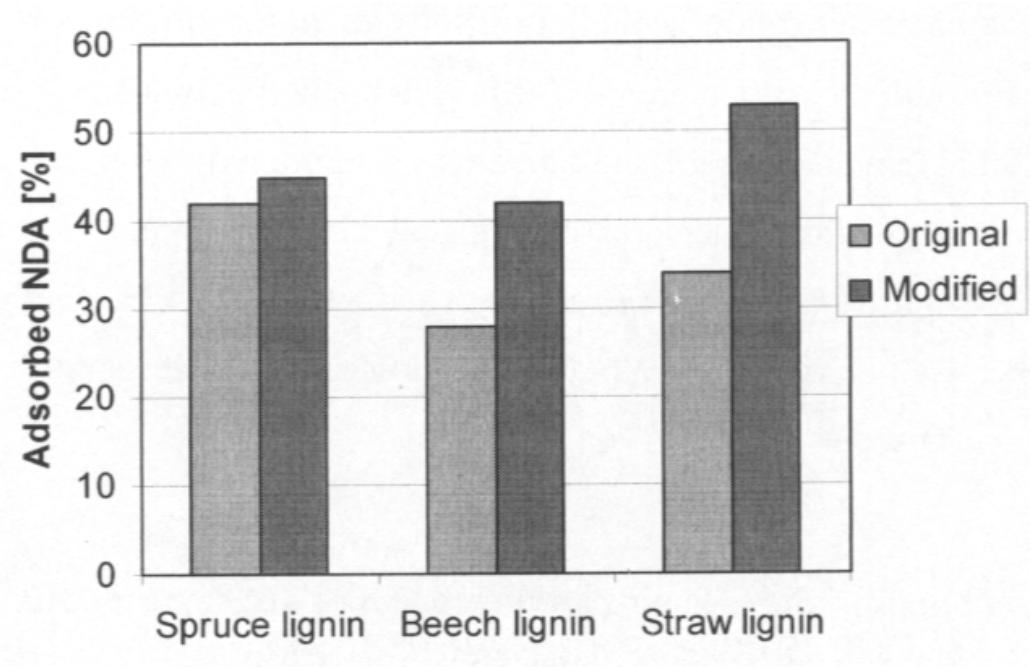

Fig.1. The binding of N-nitrosodiethylamine to hydrolysis spruce, beech, and straw lignins before and after modification

The antimutagenic effects of the prepared lignin samples on MNNG-induced mutagenic lesion were examined in hamster cells V79 *in vitro.* The results of mutagenicity testing are presented in Fig. 2, where the levels of 6-TG^r mutations per 10^5 survival of hamster cells are shown. Preincubation of cells with lignin 2 reduced the level of induced mutations by about 50 %. It is evident that the lignin tested exhibited a very significant antimutagenic protective effect on the level of TG mutations induced with MNNG in hamster cells.

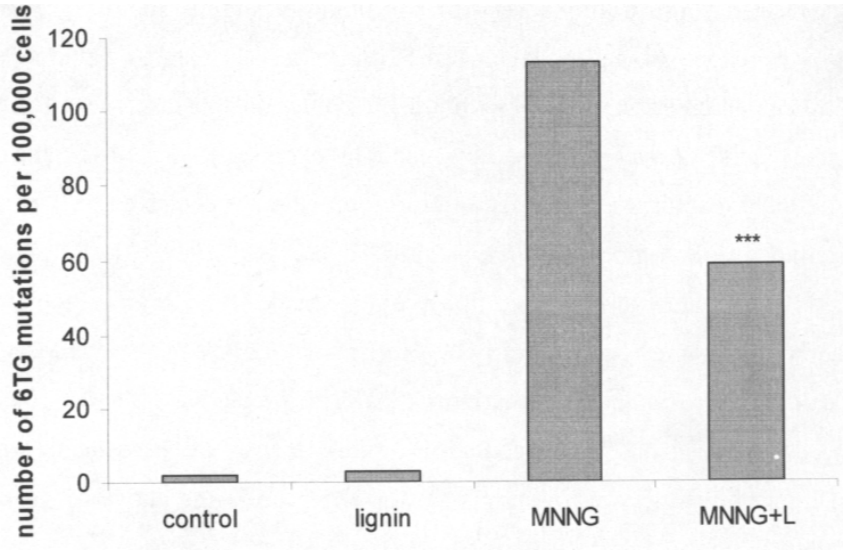

Fig. 2. Effect of lignin pre-treatment on the level of 6-TG^r mutations induced in hamster cells V79 with N-methyl-N-nitro-N-nitrosoguanidine (MNNG)

Figure 3 represents the protective effect of chemically (lignin1) and biologically modified samples (lignins 2 and 3) against DNA lesions induced in hamster cells by MNNG and scored by the comet assay. All lignin preparations significantly decreased the level of DNA strand breaks.

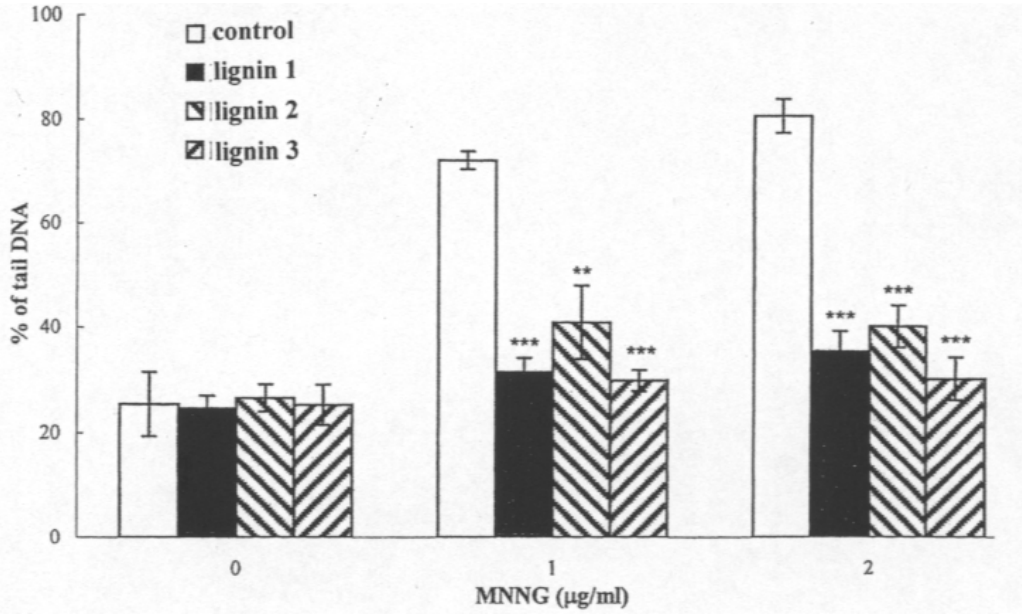

Fig. 3. Influence of lignin samples on the level DNA lesions induced by N-methyl-N-nitro-N-nitrosoguanidine in hamster cells V 79

The obtained results confirm the ability of all modified lignin samples in the concentration 50 μg/ml to reduce alkylation damage of DNA induced in hamster cells by N-methyl-N-nitro-N-nitrosoguanidine (MNNG). The observed reduction mutations and DNA-strand breaks induced by MNNG may be caused by reactions of mutagenic methyl radicals with lignin hydroxyl groups, resulting in methylation of lignin macromolecules as well as by binding affinity of lignins towards genotoxic N-nitrosoamins.

The ability of lignin 3 to protect organisms against the development of cancer by halogenated hydrocarbon pesticide DBCP was examined in lignin-fed rats in *ex vivo* experiments. DBCP has been reported to be mutagenic in various experimental test systems (Blum and Ames 1977). Hepatocytes were isolated from rats fed a standard diet (without lignin) and rats fed a diet containing 8 wt% lignin. The results show that lignin significantly decreased the level of DBCP-induced strand breaks in rat hepatocytes in *ex vivo* experiments (Fig. 4).

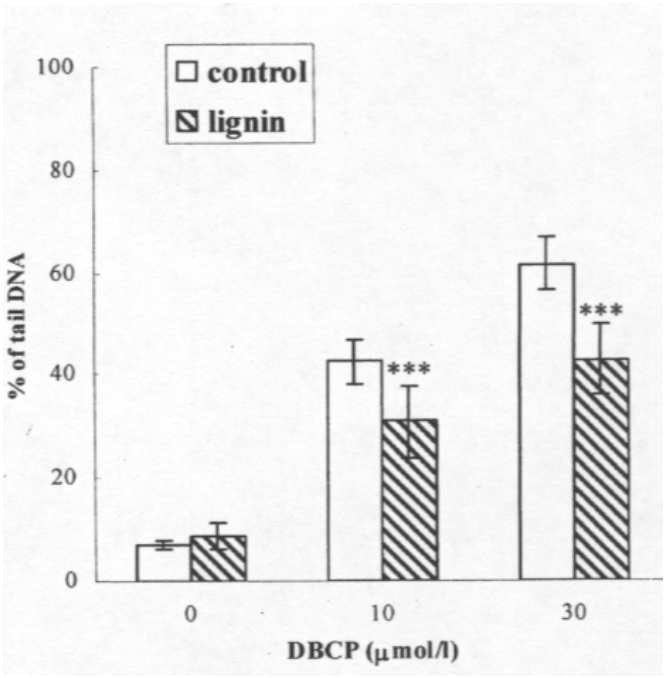

Fig. 4. Effect of dietary intake of 8 wt% lignin 3 on the level on DNA strand breaks induced by 1,2-dibromo-3-chloropropane (*ex vivo*). Values represent the mean of three independent experiments; two control rats and two lignin-fed rats were used.

This protective effect of lignin against DBCP-induced DNA lesions can be assumed to be associated with the above-described adsorption affinity of lignin. Though to date the exact mechanism in which lignin reduces genotoxic effects of chemical compounds is not known, there is substantial metabolic and experimental evidence that lignin belongs to the class of micronutrients that can decrease the risk of cancer development.

CONCLUSIONS

1. *In vitro* studies confirmed that all modified lignins exhibit comparably high binding affinity for N-nitrosoamins. In contrast, no binding ability of tested polysaccharide preparations towards NDA could be detected.
2. The revealed reduction of MNNG-induced mutations and level of DNA strand breaks in hamster cells by lignin adsorbents indicates their prospective application as natural agents for prevention of cancer and other mutation-related diseases, with potential to replace compounds prepared by organic synthesis.
3. The observed reduction of DBCP-induced DNA lesion in animals consuming a lignin-containing diet is very promising for medical applications.

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